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Expression of Bone Sialoprotein in Human Lung Cancer

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Abstract. Lung cancer belongs to the group of malignant lesions that specifically select bone as secondary implantation site. The molecular bases for this property, defined as osteotropism, is still largely unknown. The recent demonstration that human breast cancer cells express and attach to bone sialoprotein (BSP), a sulfated phosphoprotein rich in bone and other mineralized tissues, could provide a clue to elucidating bone metastases formation. BSP contains the integrin binding peptide Arg-Gly-Asp (RGD), as well as non-RGD cell attachment domain. Using an immunoperoxidase technique and a specific polyclonal antibody directed against a BSP synthetic peptide, we examined the expression of BSP in 48 lung lesions including 25 squamous carcinoma, 21 adenocarcinoma, and 2 bronchioloalveolar cancers, as well as 38 human ovarian carcinoma that constitute a group of generally nonosteotropic cancers. BSP was not specifically detected in normal lung tissue with the exception of cartilage associated with bronchi. Most of the adenocarcinoma (74%) and all squamous carcinoma of the lung examined exhibited detectable levels of BSP. Staining was mainly cytoplasmic and membrane associated. The two bronchioloalveolar lung cancers examined did not show detectable amounts of BSP. When microcalcifications were observed in pulmonary malignant lesions, they were usually associated with cancer cells expressing BSP. Only 21% of the ovarian cancers examined contained malignant cells with 2+ or 3+ positivity for BSP. We further demonstrated that in 8 of 10 additional lung cancers, BSP was detected at the mRNA level. Our observation is the first demonstration that BSP is expressed in non-small cell lung carcinoma. Lung cancer cells are now the second type of osteotropic malignant cells described to express BSP. Added to the observation that BSP expression is not frequent in ovarian carcinoma, a low osteotropic cancer, our study supports our hypothesis that BSP could play a role in determining the affinity of cancer cells to bone.

Key words: Bone sialoprotein — Lung cancer — Bone metastasis — Microcalcifications.

Lung cancer is among the few types of cancers that account for the majority of bone metastases. In patients with lung cancer, bone metastases are responsible for significant morbidity due to pain, pathological fractures, hypercalcemia, and bone marrow replacement [1–3] The pathophysiology of bone metastases is poorly understood. Aside from lung cancer cells, malignant cells originating from breast, thyroid, prostate, and myeloma exhibit a selective affinity for bone [4]. Cells from other malignancies such as ovarian or colon cancers do not colonize bone very frequently. The molecular bases for the osteotropism of metastatic cells remain to be unveiled. The recent demonstration that bone sialoprotein (BSP), a bone matrix protein, is expressed by human breast cancer cells, could provide a clue to this phenomenon [5]. BSP constitutes 10–15% of the noncollagenous proteins in the mineral compartment of bone [6]. It is a phosphorylated and sulfated sialoprotein that contains several clusters of consecutive glutamic acid residues all of which may contribute to its strong interaction with hydroxyapatite bone crystal [7]. BSP contains an RGD (Arg-Gly-Asp) motif [8], a cell attachment recognition consensus sequence for several adhesion receptors of the integrin family [9, 10]. A recent report suggests that osteoclasts attach to BSP using the integrin receptor $\alpha v\beta 3$ [11]. BSP has been detected in mineralized collagen matrices such as bone and dentine [6], cementum [12], and calcified cartilage of the growth plate [13]. BSP expression has also been reported in human trophoblastic cells [13] and to a lesser extent in decidua [8]. Our recent finding that human breast cancer cells express BSP was unexpected [5]. In our previous report, we found an association between BSP expression by breast cancer cells and the presence of microcalcifications within the lesion, suggesting that BSP could participate with hydroxyapatite deposition in malignant breast lesions [5]. Moreover, we have recently shown that BSP expression in breast cancer is associated with an increased risk for the patient to develop bone metastases [14]. Although the biological significance of BSP expression in human breast cancer remains to be elucidated, our results suggest that BSP could play a role in the bone homing of metastatic breast cancer cells. If BSP plays a significant role in the genesis of bone metastases, then this glycophosphoprotein should be detected in other malignant lesions that exhibit high osteotropism. Moreover, BSP expression should not be a common feature of non-osteotropic cancer cells. In this report, we have examined by immunoperoxidase and RT-PCR coupled to Southern blotting, the possibility that BSP could be detected in lung cancer, one of the few cancers that preferentially metastasize to bone. We have also examined by immunoperoxidase the expression of BSP in a series of human ovarian carcinoma.

Material and Methods

Tissue Specimens

A total of 48 human lung cancer tissues were obtained from the Istituto dei Tumori, Milan, Italy. Specimens were fixed in forma-

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lin, embedded in paraffin, and cut into fine sections. The human lung tissues examined included 25 squamous carcinoma, 21 adenocarcinoma, and 2 bronchioloalveolar cancers. Adjacent normal lung tissue was examined when present. Frozen tissues from 10 additional lung carcinomas were also used for BSP mRNA determination. Paraffin-embedded slides of 38 primary epithelial ovarian carcinoma samples were obtained from Ospedale San Gerardo, Monza and Clinica Valduce, Como, Italy.

Immunohistochemistry

Bone sialoprotein was identified by the avidin-biotin peroxidase complex (ABC) method using LF83 rabbit polyclonal antibody. LF83 was generated using a synthetic peptide of human bone sialoprotein (residues 277–294), which has been shown to detect the epitope RAYED [15]. This antibody has been previously checked for reactivity by Western blot and is not known to react with any protein other than BSP. Immunoperoxidase was performed using the ABC Vectastain ''Elite'' kit (Vector Laboratories, Burlingame, CA) according to the supplier's protocol. Briefly, tissue sections were deparaffinized in xylene and hydrated in phosphate-buffered saline (PBS, 10 mM sodium phosphate, 0.9% NaCl, pH 7.5). Blocking of the endogenous peroxidase was performed with 0.3% H_2O_2 in methanol and the nonspecific serum-binding sites were blocked with normal goat serum (1:20, Vector). Anti-BSP LF83 at a dilution of 1:1000 was applied and incubated for 2 hours at room temperature. Then, the tissue sections were incubated with biotinylated goat anti-rabbit antibody (1:200) followed by exposure to performed streptavidin-biotinylated horseradish peroxidase complex. Peroxidase was revealed by the 3, 3′- diaminobenzidine reaction. Finally, sections were counterstained with hematoxylin, dehydrated, and mounted. Negative control experiments included omission of the first antibody and preincubation of LF83 antibody with an excess of the corresponding or unrelated synthetic peptides.

Evaluation of Staining

The immunohistochemically stained sections were reviewed by two independent observers. The degree of staining was evaluated using an arbitrary semiquantitative scale: (0) negative; (1+) focal areas with sparse staining or occasional individual positive cells; (2+) at least one focus with extensive staining or numerous areas with weak to moderate staining; or (3+) extensive staining of more than 50% of the neoplastic cells. The Chi-square test was used to determine the significance of potential differences among lung cancer histological groups studied.

RNA Extraction, RT-PCR Analysis, and Southern Blotting

Total RNA was isolated from 10 additional lung tumors and from UMR-106 rat osteosarcoma cell line used as positive control [16], by the guanidine isothiocyanate extraction procedure and cesium chloride gradient centrifugation [17]. The presence of BSP mRNA was demonstrated by reverse transcription reaction coupled to a polymerase chain reaction (RT-PCR). Two micrograms of total RNA from each preparation were subjected to reverse transcription using an antisense primer (5′-GCAGCCGGATCCTCAT-GCATTGGCTCCAGTGACACT-3′), followed by amplification by PCR with an additional primer (5′-GGAATTCTGCTCAG-CATTTTGGGAAT-3′). Underlined bases represent non-BSP sequences that could have been used to subclone any bands of unusual size. For Southern blotting, 30 μl of the PCR products were fractionated by electrophoresis on a 1% agarose gel. The DNA was denatured, neutralized, and transferred to a nylon Hybond N+ membrane (Amersham International, UK) according to the supplier's protocol. The membrane was hybridized with a ^{32}P-labeled probe, prepared by random priming (Boehringer, Mannheim, Germany), of human BSP cDNA (clone B6-5g) [8]. Hybridization was performed according to Church and Gilbert [18] at 65°C in 0.5 M $NaPO_4$ (pH 7.2), 1 mM EDTA, 1% BSA, and 7% SDS. Filters were washed at 65°C in 40 mM $NaPO_4$, 1 mM EDTA, and 1% SDS, and autoradiographed on X-ray films at −70°C with intensifying screens.

Results

The expression of BSP was examined in 48 human lung cancers and 38 ovarian carcinoma specimens using immunoperoxidase. Cartilage tissue associated with bronchi was stained by LF83 antibodies (Fig. 1A). The staining was mainly localized in the extracellular matrix of the cartilage tissue. Positivity of bronchial cartilage was considered as an internal positive control when available on the tissue sections. Normal lung tissue was found to be consistently negative or exhibiting very low levels of BSP, as shown in Figure 1B. A staining of the ciliated cells from the bronchial epithelium was observed. However, this staining was not specific since, contrarily to cartilage staining, it was not abolished by pre-incubation of LF83 anti-BSP antibody with an excess of corresponding synthetic peptide (data not shown). Most human lung cancers examined expressed detectable levels of BSP (Table 1). All squamous lung carcinoma included in this study exhibited detectable levels of BSP with 56% of the lesions expressing 2+ or 3+ levels of the bone matrix protein (Fig. 1D, E). Seventy-four percent of the lung adenocarcinoma analyzed were also positive for BSP (Fig. 1C, F) whereas both bronchioloalveolar cancers examined were negative. Staining was mainly cytoplasmic and membrane associated (Fig. 2C). It was specific since preincubation of the anti-BSP peptide antibody with an excess of the corresponding peptide but not with an unrelated peptide abolished the staining (Fig. 2D). Usually, expression of BSP was heterogenous within the same lesion (Fig. 1C, E). Microcalcifications were observed in 11 of the 48 lung cancers examined. Nine of the 11 lesions positive for microcalcifications expressed detectable amounts of BSP (Fig. 1D). There were no significant differences in BSP expression between adenocarcinoma and squamous carcinoma of the lung that was analyzed.

To demonstrate that BSP is expressed at the mRNA level in lung cancer, we examined 10 additional lung adenocarcinoma tissues from which we extracted RNA through reverse-transcription PCR assay (RT-PCR). Southern blot analysis performed on RT-PCR products and probed with labeled human BSP cDNA was used to demonstrate that the amplified DNA fragments correspond to BSP. A single 627 bp DNA fragment that hybridized specifically, with variable intensity, to BSP cDNA probe was observed in 8 of the 10 lung tumors analyzed (Fig. 3). The same DNA fragment was also detected in UMR-106 rat osteosarcoma cells used as positive control whereas no bands were observed with the negative control consisting of PCR reaction performed on nonreverse transcripted RNA.

We have also analyzed BSP expression in ovarian carcinoma which is generally a nonosteotropic cancer. In fact, the incidence of bone metastases identified at autopsy studies of patients dying of ovarian cancer is reported to be only 9% [19]. Using the same anti-BSP antibody, only 8 of 38 (21%) of the ovarian carcinoma analyzed showed detectable levels of BSP evaluated as (2+ or 3+) whereas the majority of these lesions showed no (Fig. 2A) or barely detectable (Fig. 2B) immunoreactivity to anti-BSP antibodies.

Discussion

Until recently, BSP was considered as a protein whose ex-

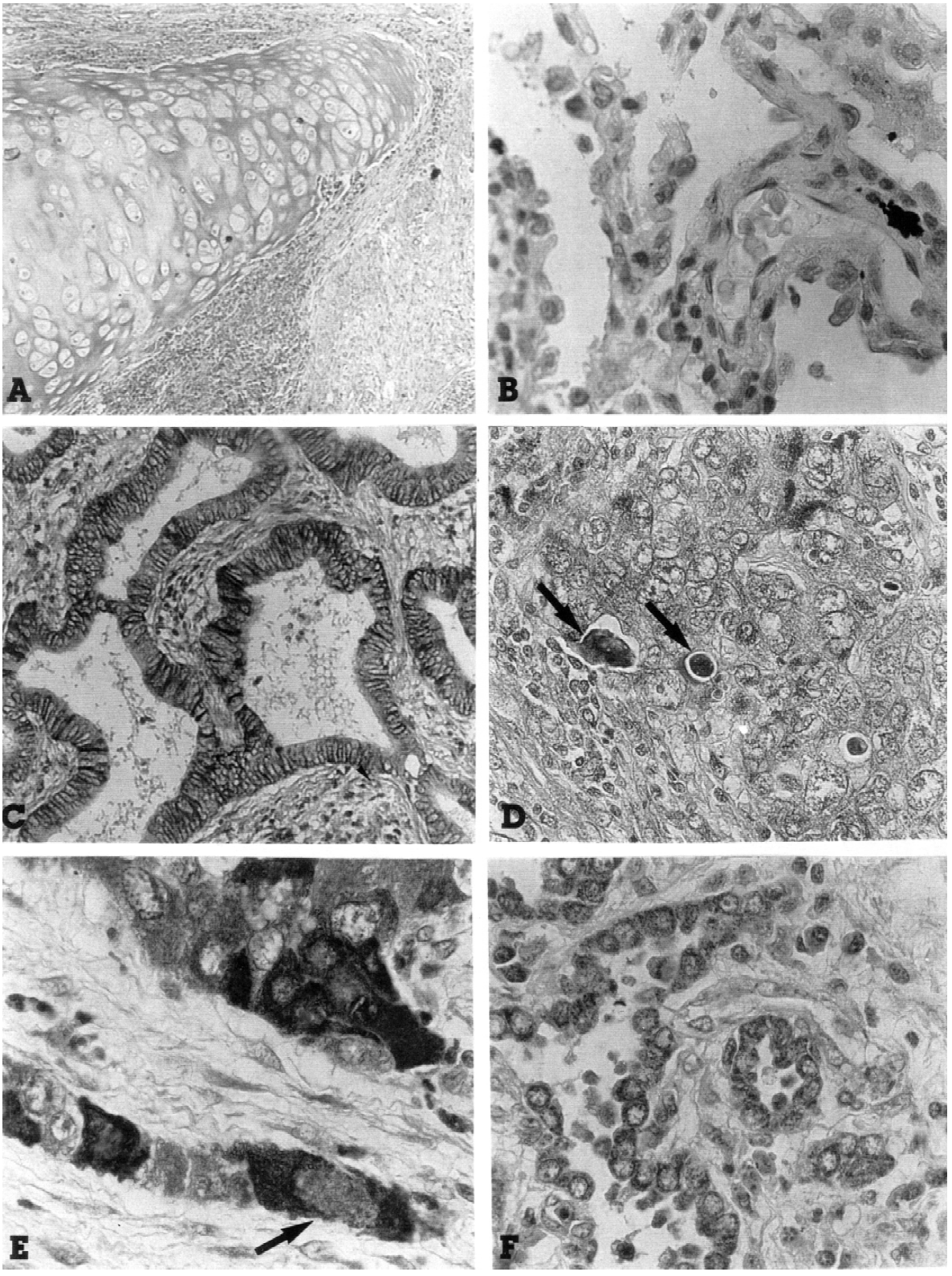

Fig. 1. Immunostaining of bone sialoprotein in human lung cancer. Paraffin-embedded tissue sections were immunostained with LF83 polyclonal antibody and counterstained with hematoxylin as described in Materials and Methods. **(A)** Cartilage tissue associated with a bronchus expressing BSP. **(B)** Normal lung parenchyma adjacent to a malignant lesion. **(C)** Lung adenocarcinoma exhibiting an heterogeneous (1+) to (3+) level of BSP. **(D)** Squamous carcinoma of the lung with heterogeneous (1+) to (2+) BSP expression; arrows indicate microcalcifications. **(E)** Squamous lung cancer of the lung with heterogeneous (2+) to (3+) levels of BSP (arrow). **(F)** Lung adenocarcinoma showing a (3+) BSP immunoreactivity. (Original magnifications: **A,** ×100; **B, D,** and **F,** ×400; **C,** ×200; and **E,** ×630).

Table 1. Immunohistochemical analysis of 48 lung specimens with a polyclonal antibody to bone sialoprotein (LF83)

Lung lesions	n[a]	Degree of immunoreactivity			
		0	1+	2+	3+
Squamous cancers	25	0 (0)[b]	11 (44)	8 (32)	6 (24)
Adenocarcinoma	21	4 (19)	3 (14)	8 (38)	6 (29)
Bronchioloalveolar cancers	2	2 (100)	0 (0)	0 (0)	0 (0)

[a] The number of specimens analyzed in each category
[b] Numbers in parentheses indicate the percent of specimens in each category

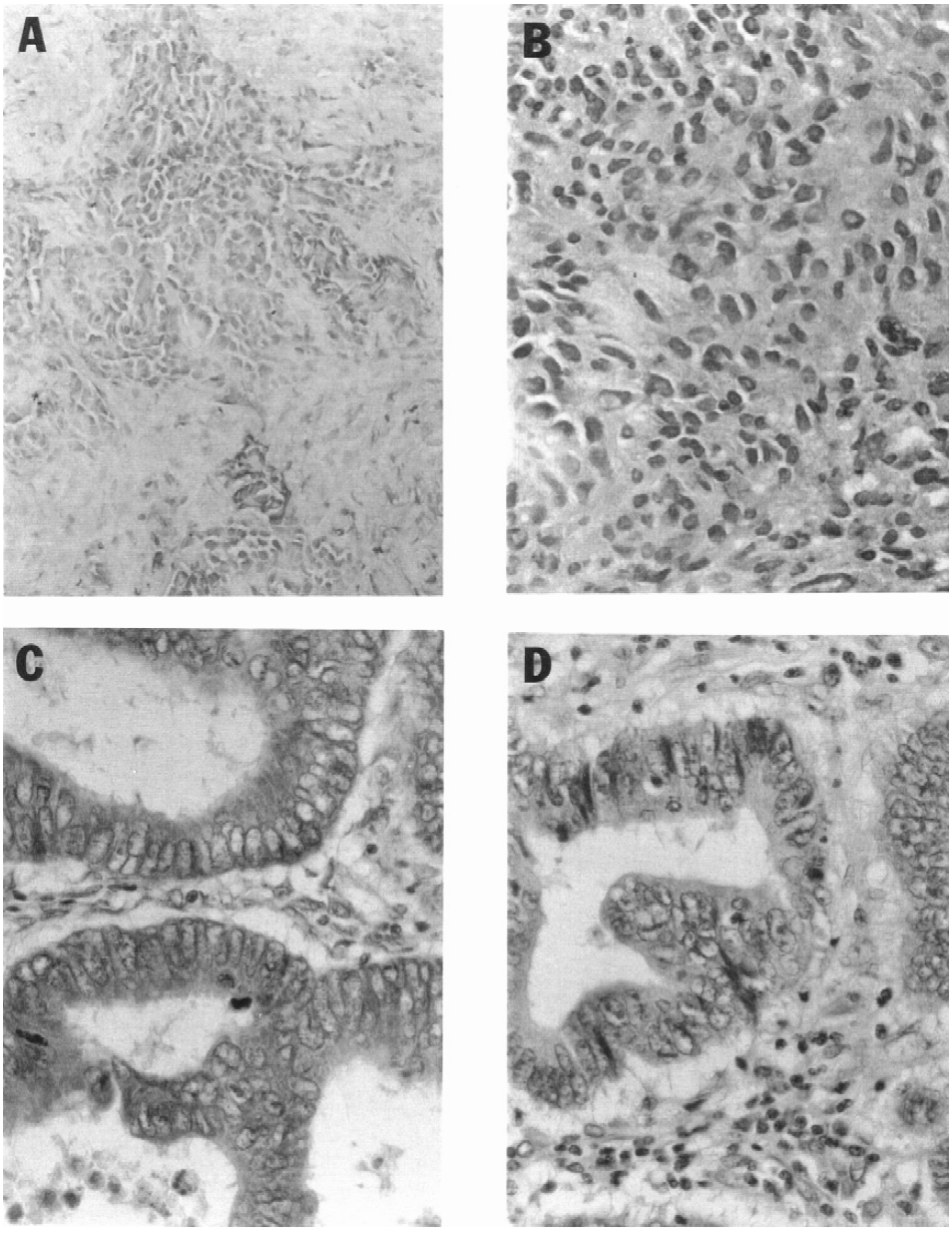

Fig. 2. Immunostaining of bone sialoprotein in human lung and ovarian cancers. Ovarian carcinoma showing no **(A)** or very low **(B)** immunoreactivity to anti-BSP (LF83) antibody. Lung adenocarcinoma tissue showing a 3+ immunoreactivity to LF83 antibody **(C).** Pre-adsorption of the antibody with the corresponding synthetic peptide results in a significant decrease of immunostaining **(D).** (Original magnification: **A** ×100; **B, C,** and **D,** ×400).

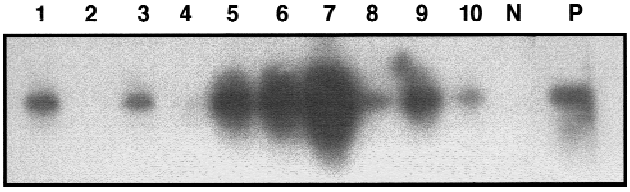

Fig. 3. Detection of BSP mRNA by RT-PCR and Southern blotting in lung cancer tissue. RT-PCR products (30 μl) from 10 lung cancer samples (lanes 1–10) and human osteoblast-like cells RNA used as positive control (lane P) were separated on a 1% agarose gel, transferred, and probed with BSP cDNA as described under Materials and Methods. Lane N represents the negative control PCR reaction product without RT.

pression was almost restricted to bone tissues [13]. Functionally, it has been proposed that BSP is involved in the initiation of mineralization of bone matrix by triggering hydroxyapatite crystallization [20]. Knockout mice lacking BSP expression show bone abnormalities, adding weight to a critical role of this phosphoprotein in the regulation of bone formation *in vivo* [21]. The demonstration that BSP promotes adhesion of osteoclasts to hydroxyapatite indicates that this phosphoprotein could modulate bone resorption [11]. Interestingly, BSP is also detected in trophoblastic cells which in late term human placenta also form mineralized nodules [22]. However, the biological role of this bone matrix protein during implantation has not yet been investigated. The recent demonstration of BSP expression in human breast cancer cells raised the question of a potential role for this phosphoprotein in cancer progression. We have also shown that BSP is detectable in breast cancer tissue and cell lines at both protein and mRNA levels indicating that the positivity of these cells to BSP is due to an intrinsic expression [23]. Expression of BSP in human primary breast cancer was found to be associated with an increased risk for subsequent bone metastases and a poor survival rate [16, 24]. These observations prompted us to investigate the possibility that ectopic expression of BSP could be involved in the pathogenesis of bone metastases [14]. Eighty percent of all bone metastases originate from a few types of cancers including those from breast, prostate, lung, thyroid, and multiple myeloma [19]. In this study, we demonstrate that most lung cancer specimens analyzed express BSP. We used a specific polyclonal antibody directed against BSP to demonstrate its expression in human squamous cancer and adenocarcinoma of the lung. This is the first report that shows an immunohistochemical localization of BSP in human lung cancers. The number of lesions examined in this study is too small to perform statistical analysis regarding the impact of BSP expression on survival, bone metastases development, or to correlate expression of BSP with other prognostic parameters. To answer these obvious questions, we have already launched a large retrospective study. We observed that hydroxyapatite deposits occur mainly in lung cancers with BSP-positive malignant cells. This may suggest that, as proposed for breast malignant lesions, BSP could participate in microcalcification formation in lung cancers. The detection of BSP mRNA in 8 of the 10 lung adenocarcinoma analyzed by RT-PCR and Southern blotting indicates that, as for breast cancer, lung malignant cells produce mRNA endogenously.

Lung cancer is now the second malignant condition in which expression of BSP is documented. Preliminary data indicate that BSP is expressed in prostate and thyroid carcinomas, two cancer types that also exhibit a selective affinity for the skeleton whereas the majority of ovarian cancer samples analyzed were found negative for BSP. These observations suggest that BSP could play a role in the osteotropic phenotype of metastatic cells. BSP could play such a role by promoting the attachment of malignant cells to the mineralized matrix as it has been shown for osteosarcoma cells [25], osteoclasts [11], and breast cancer cells *in vitro* [26]. Such interactions could be mediated through receptors of the integrin family. It has indeed been recently shown that invasive breast cancer cells express the αvβ3 receptor [26, 27] and that there is a selective increase in β3 integrins in breast bone secondary colonies [28]. The demonstration that BSP synthetic peptides are potent inhibitors of breast cancer cells' adhesion to bone is more evidence that metastatic cancer cells and BSP interactions through integrin receptors could be a key event in the initiation of bone metastasis.

Lung cancer is the leading cause of cancer mortality in western industrial countries, with an overall 5-year survival rate of approximately 10% [29]. Most deaths are due to local recurrence or disseminated disease. Understanding the molecular mechanisms that are responsible for the acquisition of the invasive and metastatic phenotype is a necessary step toward the development of effective therapies. Observation of the ectopic expression of BSP in lung cancer could constitute, aside from its putative prognostic value for bone metastases development, a new path of investigation for the clarification of these mechanisms.

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